

**EVALUATION OF *IN-VITRO* ANTI-INFLAMMATORY, THROMBOLYTIC AND CYTOTOXIC PROPERTIES OF *LABLAB NIGER* LEAVES**Md. Reyad-ul-Ferdous¹, Hasnin Rahman Jisha², Md. Golam Azam^{1*}¹Department of Pharmaceutical Sciences, North South University, Dhaka-1229, Bangladesh²Department of Pharmacy, State University of Bangladesh, Dhaka-1205, Bangladesh***Corresponding author e-mail:** mgazam@nothsouth.edu**ABSTRACT**

The present study was undertaken to investigate anti-inflammatory, thrombolytic and cytotoxic activities of methanol extracts of *Lablab niger* leaves and its different fractions. *In-vitro* anti-inflammatory activity of the *L. niger* leaves was evaluated by heat and hypotonic solution induced membrane stabilization method. Thrombolytic activity was evaluated using blood clot lysis model and cytotoxicity test was carried out by brine shrimp lethality bioassay. The crude methanolic extract and all tested fractions of *L. niger* leaves significantly ($p < 0.005$) protected erythrocyte membrane lysis comparable to standard aspirin, demonstrating its strong anti-inflammatory activity. In thrombolytic study, the crude methanolic extract and its different fractions demonstrated moderate to strong thrombolytic activity (25.62-40.88% lysis of human blood clot), in comparison to 66.75 % clot lysis by standard streptokinase. In the brine shrimp lethality bioassay, methanolic extract and its different fractions of *L. niger* leaves showed significant lethality with LC_{50} =value of 1.171-8.15 $\mu\text{g/mL}$, compared with vincristine sulphate (LC_{50} =0.451 $\mu\text{g/mL}$). These finding indicate that *L. niger* leaves could be a potential source of natural anti-inflammatory, thrombolytic and cytotoxic agents.

Keywords: *Lablab niger*, anti-inflammatory, thrombolytic, cytotoxic.**INTRODUCTION**

Lablab niger, commonly known as country bean is one of the major fruit vegetables from the Fabaceae, formerly Leguminosae family. It is a summer growing annual or short-lived perennial herbaceous high-yielding species widely distributed in the tropics, especially in Africa, India, Bangladesh, and Indonesia [1,2]. The plant possess a weak stem that derives support from climbing, twining, or creeping along a surface [2,3]. The leaves are 3-foliolate with the leaflets having a broad ovate-rhomboid shape measuring 7 to 15 cm long. The dorsal side of the leaf is smooth and the underside is hairy [3]. The flowers are white or pink, fascicled on nodes of lax racemes. The pods are compressed, tipped with the hooked persistent base of the style [4]. *Lablab* contains anti-nutritional factors (lectins, protease inhibitors, cyanogenic glucosides and canavanine) which make it unsuitable for monogastrics [1]. The

plant serve as an outstanding resource for tropical agricultural systems and in improving human food and animal feedstuffs [5]. To the best of our knowledge, no intensive scientific works on medicinal properties of *Lablab niger* leaves has been reported yet. In continuation of our search for anti-inflammatory, thrombolytic and cytotoxic agents of natural origin from the flora of the South Asian biodiversity region, we have investigated anti-inflammatory, thrombolytic and cytotoxic properties of crude methanol extract of *Lablab niger* leaves and its different fractions soluble in aqueous and organic solvents.

MATERIALS AND METHODS

Plant material: The leaves of *Lablab niger* were collected from Botanical garden, Mirpur, Dhaka, Bangladesh in November 2012. A voucher specimen for this plant has been maintained in Bangladesh

National Herbarium, Dhaka, Bangladesh (Accession no.38616). The sun dried and powdered leaves (500 gm) of *L. niger* was macerated in 2.5 L of methanol for 7 days and then filtered through a cotton plug followed by Whatman filter paper number 1. The extract was concentrated with a rotary evaporator at low temperature (40-45 °C) and reduced pressure. The concentrated methanolic extract (ME) was fractionated by modified Kupchan partitioning method [6] and the resultant partitionates i.e., petroleum ether (PESF), carbon tetrachloride (CTCSF), chloroform (CSF), and aqueous (AQSF) soluble fractions were used for the experimental processes.

Anti-inflammatory activity: The membrane stabilization by hypotonic solution and heat-induced hemolysis method was used to assess anti-inflammatory activity of the plant extracts by following standard protocol developed by Shinde *et al.* [7] and modified by Sikder *et al.* [8]. In hypotonic solution-induced method, the test sample consisted of stock erythrocyte (RBC) suspension (0.50 mL) was mixed with 5 mL of hypotonic solution (50 mM NaCl) in 10 mM sodium phosphate buffered saline (pH 7.4) containing either the extracts (1.0 mg/mL) or aspirin (0.1 g/mL). The control sample consisted of 0.5 mL of RBCs mixed with hypotonic buffered saline alone. The mixture was incubated for 10 min at room temperature, centrifuged for 10 min at 3000 g and the absorbance of the supernatant was measured at 540 nm. The percentage inhibition of either haemolysis or membrane stabilization was calculated using the following equation –

% inhibition of haemolysis = $100 \times (OD_1 - OD_2 / OD_1)$
Where, OD_1 = optical density of control and OD_2 = optical density of test sample in hypotonic solution.

In heat-induced haemolysis, isotonic buffer containing aliquots (5 ml) of the different extracts were put into two duplicate sets of centrifuge tubes. The vehicle, in the same amount, was added to another tube as control. Erythrocyte suspension (30 μ L) was added to each tube and mixed gently by inversion. One pair of the tubes was incubated at 54 °C for 20 min in a water bath, while the other pair was maintained at (0-5) °C in an ice bath. The reaction mixture was centrifuged for 3 min at 1300 g and the absorbance of the supernatant was measured at 540 nm. The percentage inhibition or acceleration of hemolysis was calculated according to the equation:
% Inhibition of hemolysis = $100 \times [1 - (OD_2 - OD_1 / OD_3 - OD_1)]$

Where, OD_1 = optical density of unheated test sample, OD_2 = optical density of heated test sample and OD_3 = optical density of heated control sample.

Thrombolytic activity: The thrombolytic activity of all extracts was evaluated by the method developed by Dagainawala [9] and slightly modified by Kawsar *et al.* [10] using streptokinase (SK) as the standard. In short, the plant extract (100 mg) suspended in 10 mL of distilled water was kept overnight. After decantation the soluble supernatant was filtered through a 0.22-micron syringe filter. Then venous blood (500 μ L) drawn from healthy volunteers was distributed in microcentrifuge tube and incubated at 37°C for 45 minutes. After clot formation, the serum was completely removed without disturbing the clot and the clot weight was determined. 100 μ L aqueous solutions of the extract was added separately to each microcentrifuge tube with the pre-weighed clot. 100 μ L (30,000 I.U) of commercial streptokinase (SK) and 100 mg of distilled water were separately added to the control tube as positive and negative controls, respectively. All the tubes were then incubated at 37 °C for 90 minutes and observed for clot lysis. After incubation, the released fluid was removed and the difference in weight after clot disruption was calculated. The difference in weight before and after clot lysis was expressed as percentage of clot lysis as shown below:

% of clot lysis = (weight of released clot / clot weight) $\times 100$

Brine shrimp lethality (BSL) bioassay: The BST bioassay was performed according to the procedure described by Meyer *et al.* [11] and modified by MacLaughlin *et al.* [12]. For the experiment, different concentrations (400-0.781 μ g/mL) of methanol extract and its petroleum ether, carbon tetrachloride, chloroform and aqueous soluble fractions were prepared in DMSO. General toxic property of the plant extract and its different fractions against *Artemia salina* was determined in a 1-day *in vivo* assay. Vincristine sulphate was used as positive control. The mortality percentage and LC_{50} (lethal concentration for 50% of the population) were determined using statistical analysis and the graph plotted concentration against percent lethality.

Statistical analysis: The results were expressed as the mean \pm standard deviation (SD). Statistical significance of the mean mortality at each concentration was analysed using one-way analysis of variance (ANOVA) and compared using Duncan's multiple range test. Values of $p \leq 0.05$ were taken to be statistically significant.

RESULTS AND DISCUSSION

Anti-inflammatory activity: The *in vitro* anti-inflammatory activity of methanol extracts was

studied by the membrane stabilization method and results are shown in Table 1. At 1.0 mg/ml, different fractions of the plant significantly protected lysis of erythrocyte membrane which was comparable to the standard aspirin. In hypotonic solution induced condition, highest (64.85 %) protection of erythrocyte membrane was found in CSF, followed by CTCSF (55.98 %) and AQSF (53.02 %). Methanol extract and the pet-ether soluble fraction (PESF) inhibited 48.99 % and 43.04% haemolysis of RBCs, respectively. In heat induced condition, methanol extract and different solvents soluble fractions of *L. niger* inhibited 25.07 % and 29.93 - 57.45 % hemolysis of RBC, respectively as compared to 42.12% hemolysis inhibited by aspirin. Prevention of hypotonicity and heat induced red blood cell membrane lysis was taken as a measure of the mechanism of anti-inflammatory effect of the plant extract since human red blood cell (HRBC) membranes are similar to lysosomal membrane components [13]. Membrane stabilization results in prevention of leakage of serum proteins and fluids into the tissues during a period of increased permeability caused by inflammatory mediators [14]. The results showed that different extractives of the plant protect the erythrocyte membrane against by both heat and hypotonic solution induced lysis. The anti-inflammatory activity of the plant was comparable to that of aspirin. The anti-inflammatory activity may be due to the inhibitory effect on enzymes involved in the production of the chemical mediators of inflammation and metabolism of arachidonic acid [15].

Thrombolytic activity: *L. niger* leaves extracts were assessed for thrombolytic activity using a simple and rapid *in-vitro* clot lysis model and the results are presented in Figure 1. The methanol extract of the plant exhibited highest thrombolytic activity (40.88% lysis of clot). Different fractions such as CSF, CTCSF, AQSF and PESF also demonstrated significant lysis of clot (37.20%, 28.24%, 25.62% and 23.12%,

respectively), in comparison to 66.75% clot lysis by the positive control SK (30,000 I.U.) and 3.02% clot lysis by water. The thrombolytic activity is probably due to the plant's diverse composition like flavonoids, tannins and terpenoids [16].

Brine shrimp lethality bioassay: Mortality and the LC₅₀ values of the different extracts of *L. niger* leaves were compared to that of VS (LC₅₀ = 0.451 µg/mL). The BSL bioassay indicated that CTCSF was the most active among all tested fractions, having an LC₅₀ of 1.71 µg/mL (Figure 2). ME showed moderate activity (LC₅₀ = 3.39 µg/mL) and other fractions were also found to be significantly bioactive (LC₅₀ = 5.11 - 12.04 µg/mL). The brine shrimp test represents a simple, rapid and inexpensive bioassay for testing plant extract lethality which in most cases has a good correlation with cytotoxic and anti-tumour properties [17,18]. Crude extracts resulting in LC₅₀ values of less than 250 µg/mL is usually considered significantly active and potential for further investigation [19]. The result obtained from the brine shrimp lethality bioassay of *L. niger* can serve as a guide for the isolation of cytotoxic compounds from the methanol extract and different fractions of the plant by using more specific and more sophisticated bioassays.

CONCLUSION

Results of the present study for the first time indicate that the *L. niger* leaves possess significant anti-inflammatory, thrombolytic and cytotoxic properties. Different extract and fractions can therefore be regarded as a safe, economic natural source for the discovery of new anti-inflammatory, thrombolytic and cytotoxic agents. Further studies on identification, isolation and purification of active principles of the plant responsible for these therapeutic properties may lead to new drug development.

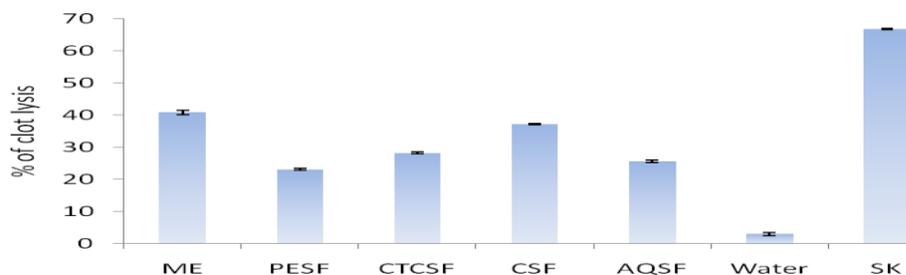


Figure 1. Thrombolytic activity of crude extract and different fractions of *L. niger*. (ME = Methanolic extract; PESF = Pet-ether soluble fraction; CTCSF = Carbon tetrachloride soluble fraction; CSF= chloroform soluble fraction; AQSF = Aqueous soluble fraction of the methanolic extract of *L. niger*. SK = Streptokinase).

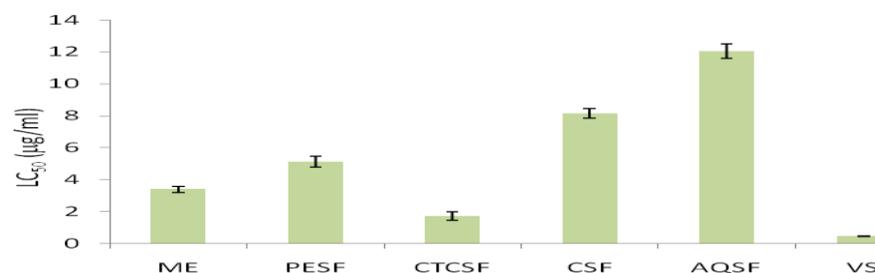


Figure 2. Brine shrimp cytotoxicity of crude extract and different fractions of *L. niger* (VS = Vincristine sulphate; ME = Methanolic extract; PESF = Pet-ether soluble fraction; CTCSF = Carbon tetrachloride soluble fraction; CSF= chloroform soluble fraction; AQSF = Aqueous soluble fraction of the methanol extract of *L. niger*).

Table-1: Effect of *L. niger* on hypotonic solution and heat induced haemolysis of erythrocyte membrane.

Test Samples	Concentration	% inhibition of haemolysis*	
		Hypotonic solution induced	Heat induced
ME	1.0 mg/mL	48.99±0.51	25.07±0.39
PESF	1.0 mg/mL	43.04±0.44	33.04±0.44
CTCSF	1.0 mg/mL	55.98±0.52	29.95±0.57
AQSF	1.0 mg/mL	53.02±0.47	57.01±0.49
CSF	1.0 mg/mL	64.85±0.25	57.45±0.50
Aspirin	0.1 mg/mL	71.8±0.26	42.2±0.26

ME = Methanolic extract; PESF = Pet-ether soluble fraction; CTCSF = Carbon tetrachloride soluble fraction; CSF= chloroform soluble fraction; AQSF = Aqueous soluble fraction of methanolic extract of *L. niger*. *P <0.005.

REFERENCES

- Lambourne LJ, Wood IM. Aust J Exp Anim Husb, 1985; 25: 169.
- Milford R, Minson D J. Aust J Exp Anim Husb, 1968; 8:409-418.
- Cameron DG. Queensland Agr J, 1988 March-April: 110-113.
- Balick JM, Cox PA. Plants, People and Culture: the Science of Ethnobotany. New York; Scientific American Library: 1996.
- Pengelly BC, Lisson SN. Strategies for Using Improved Forages to Enhance Production in Bali Cattle. In: Entwistle K and Lindsay DR (eds). Proceedings of a Workshop Australian Centre for International Agricultural Research (ACIAR), Bali: 2002, pp. 29-33.
- Van Wagenen BC, Larsen R, Cardellina JH, Ran dazzo D, Lidert ZC, Swithenbank C. J Org Chem, 1993; 58: 335-337.
- Shinde UA, Phadke AS, Nair AM, Mungantiwar AA, Dikshit VJ, Saraf MN. Fitoterapia, 1999; 70: 333-339.
- Sikder MA, Rahman MA, Kaiser MA, Rahman MS, Hasan CM, Rashid MA. Lat Am J Pharm, 2011; 30: 781-5.
- Daginawala HF, Prasad S, Kashyap RS, Deopujari JY, Purohit HJ, Taori GM. Thrombosis J, 2006; 4: 14.
- Kawsar MH, Sikder MA, Rana MS, Nimmi I, Rashid MA. Bangl Pharm J, 2011; 14: 103-106.
- Meyer BN, Ferrigni NR, Putnam JE, Jacobsen JB, Nicholsand DE, Mclaughlin JL. Planta Med, 1982; 45: 31-34.
- McLaughlin JL, Anderson JE, Rogers LL. The use of biological assays to evaluate botanicals. Drug Infor J, 1998; 32: 513-524.
- Mounnissamy VM, Kavimani S, Balu V, Drlin QS. Iranian Pharmacol Ther, 2008; 6: 235-237.
- Chaitanya R, Sandhya S, David B, Vinod KR, Murali S. Int J Res Pharm Biomed Sci, 2011; 2(1): 256-259.
- Oweyele B, Oloriegbe YY, Balaogun EA, Soladoye AO. J Ethnopharmacol, 2005; 99: 153-156.
- Dwivedi S. Terminalia arjuna Wight & Arn.-A useful drug for cardiovascular disorders. J Ethnopharmacol, 2007; 114: 114-29.
- McLaughlin JL. Methods Plant Biochem, 1991; 6: 1-32.
- Mclaughlin JL, Rogers LL, Anderson EJ. Drug Info J, 1998; 32: 513-524,
- Rieser MJ, Gu ZM, Fang XP, Zeng L, Wood KV, McLaughlin JL. J Nat Prod, 1996; 59: 100-108.